Proportions of Myosin Heavy Chain *mRNAs*, Protein Isoforms and Fiber Types in the Slow and Fast Skeletal Muscles Are Maintained After Alterations of Thyroid Status in Rats

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Summary

Recently, we have established that slow soleus (SOL) and fast extensor digitorum longus (EDL) muscles of euthyroid (EU) Lewis rats posses the same proportions between their four myosin heavy chain (MyHC) *mRNAs*, protein isoforms and fiber types as determined by real time RT-PCR, SDS-PAGE and 2-D stereological fiber type analysis, respectively. In the present paper we investigated if these proportions are maintained in adult Lewis rats with hyperthyroid (HT) and hypothyroid (HY) status. Although HT and HY states change MyHC isoform expression, results from all three methods showed that proportion between *MyHC mRNA-1, -2a, -2x/d, -2b*, protein isoforms MyHC-1, -2a, -2x/d, -2b and to lesser extent also fiber types 1, 2A, 2X/D, 2B were preserved in both SOL and EDL muscles. Furthermore, in the SOL muscle *mRNA* expression of slow *MyHC-1* remained up to three orders higher compared to fast *MyHC* transcripts, which explains the predominance of MyHC-1 isoform and fiber type 1 even in HT rats. Although HT status led in the SOL to increased expression of *MyHC-2a mRNA,* MyHC-2a isoform and 2A fibers, it preserved extremely low expression of *MyHC-2x* and *-2b mRNA* and protein isoforms, which explains the absence of pure 2X/D and 2B fibers. HY status, on the other hand, almost completely abolished expression of all three fast *MyHC mRNAs*, MyHC protein isoforms and fast fiber types in the SOL muscle. Our data present evidence that a correlation between *mRNA*, protein content and fiber type composition found in EU status is also preserved in HT and HY rats.

Key words

Thyroid hormones • Muscle gene expression, MyHC isoforms and muscle fiber types • Quantitative real time RT-PCR, SDS-PAGE and 2-D stereological analysis

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Introduction

Skeletal muscle fiber types are to the great extent defined by myosin heavy chain (MyHC) protein isoform composition, which is dependent on the specific *MyHC mRNA* content and it was shown that in the rat both the EDL and SOL muscles express all four *MyHC-1, -2a, -2x/d* and *-2b mRNAs* (Soukup *et al.* 2002, 2009, Smerdu and Soukup 2008, Zurmanova *et al.* 2008, Zurmanova and Soukup 2013, for review see Soukup and Jirmanová 2000, Schiaffino 2010). We have, however, recently shown that in the SOL muscle of adult Lewis rats, level of slow *MyHC-1 mRNA* is up to three orders higher compared to fast *MyHC mRNAs* (Zurmanova and Soukup 2013). We also established that in EU Lewis rats both slow twitch SOL and fast twitch EDL posses the same proportions between their *MyHC mRNAs*, protein isoforms and fiber types determined by RT-PCR, SDS-PAGE and 2-D stereological fiber type analysis (Zurmanova and Soukup 2013). Thyroid hormones (TH) are known as powerful regulators of MyHC isoform expression and their altered levels are one of the important pathophysiological factors leading to MyHC transitions in cardiac and skeletal muscles. Both muscle types react to TH alteration by modifying their *MyHC mRNA* expression, protein isoform content and fiber type

composition (Ianuzzo *et al*. 1977, d´Albis and Butler-Browne 1993, Larsson *et al*. 1994, Caiozzo *et al*. 1997, Hudecova *et al.* 2004, Vadaszova and Soukup 2006a,b, Vadaszova-Soukup and Soukup 2007, Novák *et al.* 2010, Arnostova *et al.* 2011, for review see e.g. Schiaffino and Reggiani 1996, Pette *et al.* 1999, Soukup and Jirmanová 2000, Pette 2002, Vadaszova *et al.* 2004, Tribulova *et al*. 2010, Novák and Soukup 2011). TH as transcriptional factors acting *via* TH response element (Yen 2001) stimulate expression of fast genes and thus levels of fast MyHC isoforms and consequently fast fibers, while decreased TH levels lead to opposite changes.

The aim of the present paper was to analyze proportions in the expression of MyHC at *mRNA* and protein levels as well as fiber type composition using real time RT-PCR, SDS-PAGE and 2-D stereological fiber type analysis of immunohistochemically stained muscle cross sections in the SOL and EDL muscles of adult HT and HY Lewis rats in order to answer the question if these proportions remain preserved in rats with altered TH status, similarly as was shown in EU rats (Zurmanova and Soukup 2013).

Materials and Methods

Animals

Experiments were performed on 9 HT and 9 HY adult (average age 14 months) inbred Lewis strain rats of both sexes obtained from the authorized rat-breeding unit of the Institute of Physiology, v.v.i., AS CR, Prague (Accreditation No. 1020/491/A/00). The maintenance and handling of experimental animals were in accordance with the 2010/63/EU Directive and the investigation was approved by the Expert Committee of the Institute of Physiology, v.v.i. Results of littermate EU animals were published earlier (Zurmanova and Soukup 2013) and are used in this paper for comparison with permission of Physiological Research journal. The animals were anesthetized with intraperitoneal injections of 1 ml (100 mg) of Narketan (Ketaminum ut hydrochloridum, Vetoquinol S.A., France) per 1000 g of body weight, followed by 0.5 ml (10 mg) of the myorelaxant Xylapan (Xylazinum ut hydrochloridum, Vetoquinol Biowet, Poland) per 1000 g of body weight and sacrificed by exsanguination. The experimental muscles excised from hind limbs were frozen in liquid nitrogen, the right muscles were used for real time RT-PCR, while the left muscles were used to prepare cryocut sections or stored at –80 °C until used for SDS-PAGE.

Alteration of TH status

HT or HY states were induced in 2-month-old animals and maintained for about 12 months by either intraperitoneal injections of 3,3',5-triiodo-L-thyronine (sodium salt, T_3, 0.15 μg/kg body weight) three times a week or with a 0.05 % solution of methimazole (2-mercapto-1-methylimidazole) in drinking water, respectively. The EU rats were age-matched littermates of the HT and HY animals. The effect of our procedure was checked by measuring biochemical and anatomical parameters that are known to be influenced by TH level alterations (Soukup *et al*. 2001, Rauchová *et al.* 2004, 2011, 2013, Pavelka 2014).

RNA isolation and quantitative real time RT-PCR (qRT-PCR)

The primers described earlier (Zurmanova *et al.* 2007, 2008, Zurmanova and Soukup 2013) were designed using the Gene Runner program (Hastings Software). Total cellular *RNA* was extracted using the RNAzol RT (Sigma Aldrich), converted to cDNA using the RevertAid^{TM} H Mius First Strand cDNA Synthesis Kit (Fermentas), samples of 1 μl cDNA were amplified in 10 μl of PCR reaction mixture containing iQ^{TM} SYBR Green Supermix (Bio-Rad) and PCRs were performed on a Light Cycler (Roche Ltd.) (for a detailed description see Zurmanova *et al.* 2008, Zurmanova and Soukup 2013, Waskova-Arnostova *et al.* 2013).

Immunohistochemistry

Muscle fiber types were determined using mouse monoclonal antibodies (mAbs) specific for rat MyHC isoforms BA-D5 (MyHC-1), SC-71 (MyHC-2a), BF-35 (all MyHC except -2x/d) and BF-F3 (MyHC-2b) (Schiaffino 2010). Primary antibody binding was revealed using donkey secondary antibody conjugated with HRP (Jackson Immunoresearch Laboratories, USA) (for a detailed description see Smerdu and Soukup 2008, Soukup *et al.* 2009, 2012, Zurmanova and Soukup 2013).

SDS-PAGE

MyHC isoforms were separated according to Talmadge and Roy (1993) using MiniPROTEAN3 Cell (Bio-Rad Ltd.) as described earlier (Říčný and Soukup 2011, Zurmanova and Soukup 2013). The silver-stained gels (Blum *et al.* 1987) were densitometrically evaluated using the LAS-1000 imaging system (Fujilab, Japan) and the AIDA 3.28 computer program at two gels from each sample.

Quantitative morphological analysis

The numerical (N) proportions (%) of muscle fiber types were assessed by 2-D stereological methods using the principles of an unbiased counting frame and point counting (Zacharova and Kubínová 1995) using the C.A.S.T. Grid System (Olympus, Albertslund, Denmark). The particular arrangement of the stereological system (number of points, size of the counting frame, scanning interval) was selected according to muscle section size and fiber composition on the basis of efficacy analysis described previously (Zacharova and Kubínová 1995, Zacharova *et al*. 1997, 1999, 2005).

Statistical analysis

The data are expressed as mean ± SE. Differences between individual *MyHC* transcript level, MyHC protein isoform content and fiber type composition between TH states were evaluated using the Kruskal-Wallis One Way Analysis of Variance on Ranks (SigmaStat program, Systat Software, Germany).

Results

The MyHC mRNAs analysis by qRT-PCR

Our measurements revealed in the SOL muscles that the expression of faster *MyHC-2a* transcripts in HT status was significantly higher compared to HY (and EU) status (Fig. 1A). This increase was at the expense of slow *MyHC-1 mRNA,* because an increase of *MyHC-2x/d* was low (but significant) and *MyHC-2b mRNA* expression was negligible in both HT and HY status (Figs 1A and 2A). Similarly as in the EU rats, the level of *MyHC-1 mRNA* transcript was up to three orders higher than that of fast isoforms. On the other hand, HY status almost completely abolished expression of all three fast *mRNAs* (Figs 1A and 2A) and thus the level of *MyHC-1 mRNA* expression was almost 2000 times higher compared to the *mRNAs* of fast *MyHC* isoforms (Fig. 1A).

The differences in expression among *MyHC* transcripts in the EDL muscles were usually within the range of about one order. The highest expression levels in both HT and HY states (similarly as in EU status) showed fast *MyHC-2b* and *-2x/d* transcripts highly exceeding the levels of slower *MyHC-2a* and *MyHC-1* transcripts (Figs 1B and 3A). HT compared to HY (and EU) status significantly decreased the expression of slow *MyHC-1* and *MyHC-2a mRNAs,* while HY status led to opposite changes. Transcript level of MyHC-2x/d was lower in HY compared to HT status, opposite to MyHC-2b transcript level (Figs 1B and 3A).

The MyHC protein analysis by SDS-PAGE

HT status in the SOL muscles compared to HY (and EU) status significantly increased content of MyHC-2a isoform and decreased that of MyHC-1 isoform, while traces (1 to 6 %) of MyHC-2x/d and/or of -2b isoforms found in some muscles were not significantly changed (Fig. 2B). On the other hand, HY status further increased the dominance of MyHC-1 and almost eliminated presence of fast isoforms including MyHC-2a (Fig. 2B).

In the EDL, HT status, compared to HY (and EU) status, significantly decreased content of MyHC-1 and -2a, while rather increased content of MyHC-2x/d and -2b isoforms (Fig. 3B). HY status lead to the opposite changes (Fig. 3B).

Fiber type immunochemical analysis

In the SOL muscles, HT status significantly decreased proportion of type 1 and increased that of 2A (including hybrid 1/2A) fibers compared to HY (and EU) status, while HY status eliminated the presence of pure 2A fibers (Fig. 2C) that were replaced by a variable low number of hybrid 1/2A fibers stained by BA-D5 and SC-71 mAbs. All fibers in the SOL muscles in all TH states were stained by BF-35 mAb demonstrating the absence of pure 2X/D fibers, furthermore no fibers were stained by BF-F3 mAb specifically recognizing 2B fibers excluding presence of pure 2B fibers (Fig. 2C).

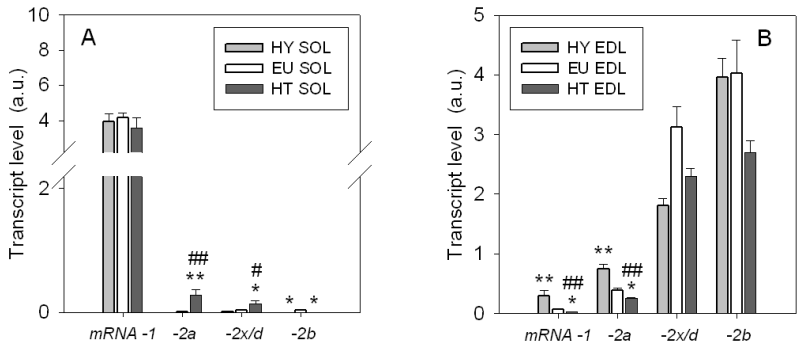

Fig. 1. A, B. Expression in arbitrary units (a.u.) of slow *MyHC-1 mRNA (MYH7 gene)* and fast *MyHC-2a (MYH2 gene), MyHC-2x/d (MYH1gene)* and *MyHC-2b (MYH4 gene) mRNAs* in the soleus (SOL) and the extensor digitorum longus (EDL) muscles of adult Lewis strain hypothyroid (hatched columns, HY), euthyroid (blank columns, EU) and hyperthyroid (dark column, HT) rats. Note the great difference between *MyHC-1 mRNA* and fast *MYH genes* levels in the SOL compared to minor differences present in the EDL. Data for EU rats are from Zurmanova and Soukup (2013). Significantly different: $^{\#}$ p≤0.05, $^{\#\#}$ p≤0.01 vs. HY status, * p≤0.05, ** p≤0.01 vs. EU status.

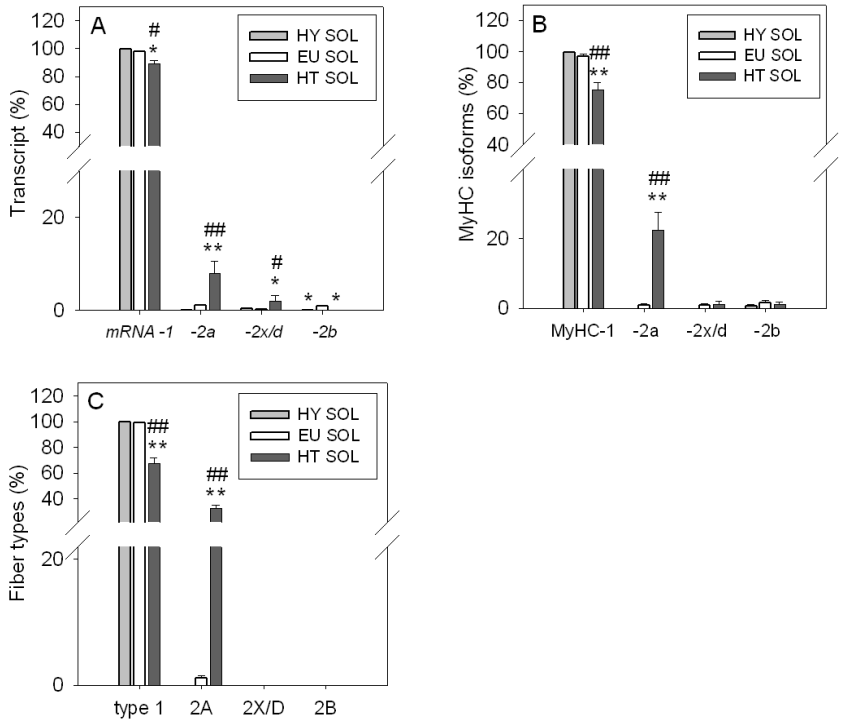

Fig. 2. A-C. Relative values (percentage) of slow *MyHC-1 mRNA* and fast *MyHC-2a, MyHC-2x/d* and *MyHC-2b mRNAs* expression as determined by qRT-PCR (Transcript), relative MyHC isoform content as determined by SDS-PAGE (MyHC isoforms) and fiber type proportions as determined by immunochemistry (Fiber types) in the soleus (SOL) muscles of adult Lewis strain hypothyroid (hatched columns, HY), euthyroid (blank columns, EU) and hyperthyroid (dark columns, HT) rats. Note the great difference in expression between the slow and fast *MyHC mRNAs* and corresponding differences in the MyHC content and fiber type proportion in all thyroid states. The close correlation between all three values indicates that MyHC isoform content and fiber phenotype are determined by gene expression and that changes observed in rats with altered thyroid status indicate regulation at the transcription level by the thyroid hormones. Data for EU rats are from Zurmanova and Soukup (2013). Significantly different: # p≤0.05, ## p≤0.01 vs. HY status, * p≤0.05, ** p≤0.01 vs. EU status.

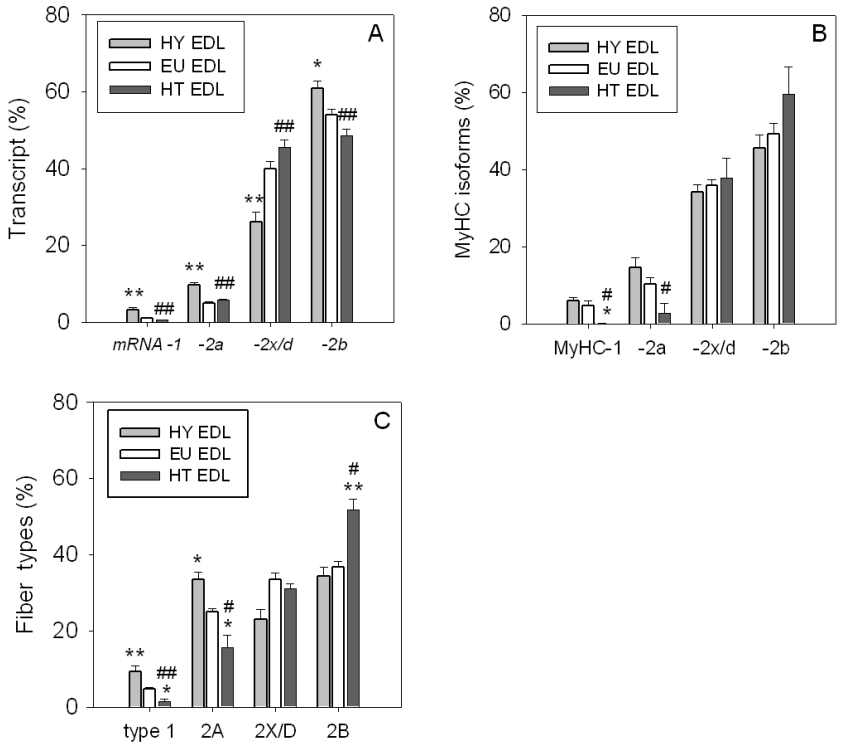

Fig. 3. A-C. Relative values (percentage) of a slow *MyHC-1 mRNA* and fast *MyHC-2a, MyHC-2x/d* and *MyHC-2b mRNAs* expression as determined by qRT-PCR (Transcript), relative MyHC isoform content as determined by SDS-PAGE (MyHC isoforms) and fiber type proportions as determined by immunochemistry (Fiber types) in the extensor digitorum longus (EDL) muscles of adult Lewis strain hypothyroid (hatched columns, HY), euthyroid (blank columns, EU) and hyperthyroid (dark columns, HT) rats. Note that the difference between *MyHC-1* and *fast* transcript levels, MyHC isoform content and fiber type proportions are much smaller compared to the soleus muscle. The close correlation between all three values indicates, similarly as in the soleus that MyHC isoform content and fiber phenotype are determined by gene expression and that changes observed in rats with altered thyroid status indicate regulation at the transcription level by the thyroid hormones. Data for EU rats are from Zurmanova and Soukup (2013). Significantly different: # p≤0.05, ## p≤0.01 vs. HY status, * p≤0.05, ** p≤0.01 vs. EU status.

The number of the fastest 2B fibers was significantly higher compared to HY (and EU) status in HT rats, while the number of 2A and type 1 fibers was significantly lower (Fig. 3C). HY status, on the other hand, significantly increased the number of 2A and type 1 fibers compared to HT (and EU) status and reduced the number of 2X/D and 2B (Fig. 3C) (for examples of immunohistochemical reactions see Zacharova *et al.* 2005, Soukup *et al.* 2012).

Discussion

Our results show for the first time that TH alterations induce corresponding changes in proportions

of MyHC *mRNAs,* protein isoforms and fiber types in HY and HT rats, similarly as was recently described in EU rats (Zurmanova and Soukup 2013).

Comparison of MyHC mRNA, protein level and fiber type composition and the role of thyroid hormones

In the slow SOL muscles of adult HT and HY Lewis rats, similarly as in their EU littermates (Zurmanova and Soukup 2013), we identified all four *MyHC-1, -2a, -2x/d* and *-2b* transcripts. The fast *MyHC-2a, -2x/d* and *-2b* transcripts were, however, up to three orders lower than that of the slow *MyHC-1* isoform, they were the lowest in HY, followed by EU and HT states. This finding correlated with the predominant content of MyHC-1 protein isoform supplemented by small amounts of fast MyHC protein isoforms, reflected by the absence of pure 2X/D and 2B fibers. Alteration of TH level resulted in significant changes in HT status, when *MyHC-2a mRNA* expression, MyHC-2a content and percentage of 2A fibers significantly increased. On the other hand, the increase of *MyHC-2x/d mRNA* compared to HY and EU states should be considered with caution because of the low expression levels. The results of immunochemical staining using BF-35 and BF-F3 mAbs showed that pure type 2X/D and 2B fibers were absent in the SOL muscles in HY and HT states, similarly as in EU status. Apparently, extremely low transcript and isoform contents are not sufficient to be represented at the phenotype level as 2X/D and 2B fibers. Although the level of all three fast transcripts was equally low, reflected by low levels of fast MyHC isoforms, 2A and or hybrid 1/2A, but not 2X/D and 2B, fibers could be detected in the majority of SOL muscles, many in HT, few in EU and exceptional in HY status. The minor increase of *MyHC-2x/d mRNA* expression in TH status did not result in increased synthesis of MyHC-2x/d and appearance of 2X/D fibers. However, as BF-35 mAb is a negative marker of 2X/D fibers, even a small amount of MyHC-1 or -2a can be stained and mask the possible existence of 2X/D fibers.

The direct correlation between *mRNA* level, MyHC protein isoform content and fiber type proportion was also revealed in the EDL muscles. The *mRNA MyHC-2b* and *-2x/d* levels were about up to one order higher compared to *mRNA MyHC-1* and even less when compared to *mRNA MyHC-2a* regardless TH status. The lowest amount of the *MyHC-1* isoform transcript corresponds to a minor representation of slow MyHC-1 isoform and of slow type 1 fibers, which is fully in agreement with results of previous studies in Lewis strain rats (Soukup *et al.* 2002, 2009, Zacharova *et al.* 2005, Smerdu and Soukup 2008, Novák *et al.* 2010, Novák and Soukup 2011, Soukup *et al.* 2012). The amount of fast *MyHC mRNAs* increased in the same order as the content of MyHC protein isoforms and fiber type percentage i.e. type 1 < 2a < 2x/d < 2b in HT as well as in EU status. In HY status, this order was preserved in the case of *MyHC mRNA* and protein isoforms, while in the case of fiber types the percentage of 2A fibers was higher than that of 2X/D and the same as that of 2B fibers. However, we suppose that due to high avidity of SC-71 antibody many fibers counted as 2A fibers were in fact hybrid 2A/X/D fibers (or even 2A/X/B). As expected, HT status increased MyHC content of the fastest -2b isoform as well as of corresponding 2B fibers, while HY status increased the proportions of MyHC-1 and -2a and of corresponding fiber types.

Three simultaneous analyses performed on the same group of animals showed that altered TH levels coordinately changed both *mRNA* and MyHC protein levels, in most cases reflected by the subsequent change of corresponding fiber types. Compared to HY status, increased TH levels (HT status) resulted in a coordinated significant increase of *mRNA MyHC-2a*, MyHC-2a isoform and 2A fibers in the SOL and their decrease in the EDL. *MyHC-2x/d* and *-2b mRNA* changes were not reflected at protein and fiber levels in the SOL, while in the EDL the two fastest isoforms usually increased. As expected, HY status coordinately increased in both muscles the expression of *MyHC-1* and *MyHC-2a mRNAs,* MyHC isoforms and corresponding fiber types and decreased that of faster isoforms (except *MyHC-2b* transcript).

Conclusions

Using three different methods we showed for the first time a direct correlation between transcript and MyHC isoform levels or fiber type proportions in corresponding muscle samples of euthyroid rats (Zurmanova and Soukup 2013). Our present results show for both SOL and EDL muscles an almost equivalent composition in percent of MyHC in the case *mRNA-1, -2a, -2x/d, -2b,* MyHC-1, -2a, -2x/d, -2b protein isoforms and type 1, 2A, 2X/D, 2B fiber types in hyperthyroid and hypothyroid rats.

Conflict of Interest

There is no conflict of interest.

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